CHEMISTRY

Nuclease-assisted selection of slow-off rate aptamers

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Conventional directed evolution methods offer the ability to select bioreceptors with high binding affinity for a specific target in terms of thermodynamic properties. However, there is a lack of analogous approaches for kinetic selection, which could yield affinity reagents that exhibit slow off-rates and thus remain tightly bound to targets for extended periods. Here, we describe an in vitro directed evolution methodology that uses the nuclease flap endonuclease 1 to achieve the efficient discovery of aptamers that have slow dissociation rates. Our nuclease-assisted selection strategy can yield specific aptamers for both small molecules and proteins with off-rates that are an order of magnitude slower relative to those obtained with conventional selection methods while still retaining excellent overall target affinity in terms of thermodynamics. This new methodology provides a generalizable approach for generating slow off-rate aptamers for diverse targets, which could, in turn, prove valuable for applications including molecular devices, bioimaging, and therapy.

INTRODUCTION

Bioreceptors such as antibodies and aptamers have revolutionized fields including medicine (*1*, *2*), forensics (*3*), biomedical engineering (*4*), and materials science (*5*) by enabling selective identification (*6*, *7*), sequestration (*8*–*10*), or functional modulation (*11*, *12*) of specific molecular targets. They are often characterized on the basis of their affinity for ligands, which is measured thermodynamically in terms of the equilibrium dissociation constant (K_d). However, there is also a growing recognition of the importance of receptor binding kinetics. For instance, in the context of therapeutics, given a set of drugs with similar K_d, those with slow drug-receptor dissociation kinetics (k_{off}) are more efficacious and potent than drugs with more rapid off-rates (*13*, *14*). Bioreceptors with slow k_{off} could also prove useful for other applications such as molecular imaging, by improving the signal-to-noise ratio (*15*), or drug delivery applications, where the timing of drug release needs to be gradual rather than immediate (*16*). Although antibodies are well established and have relatively slow off-rates, they have several disadvantages that limit their utility in these specific contexts. For instance, for in vivo imaging, the prolonged half-lives of antibodies lead to a high background, thereby reducing the signal-to-noise ratio (*17*, *18*). For drug delivery, antibodies have low tissue penetration, potentially limiting drug exposure at diseased sites (*19*). A more general issue is that because antibody generation is performed entirely in vivo, there are no means to select antibodies with a specific off-rate. Thus, antibodies must be screened individually, typically with low-throughput methods such as surface plasmon resonance (*20*, *21*).

Aptamers are a promising category of nucleic acid–based bioreceptors that can be isolated from randomized libraries through an in vitro process termed systematic evolution of ligands by exponential enrichment (SELEX) (*22*, *23*). They have several advantageous properties compared to antibodies, such as low production costs, ease of chemical modification, low batch variability, nonimmunogenicity, and high tissue penetration. One key advantage of aptamers relative to antibodies is that selection is performed entirely in vitro, such that various facets of the selection process can be manipulated to obtain aptamers with a desired set of binding characteristics (*24*). Efforts have been undertaken to isolate aptamers with slow k_{off} using a combination of strategies including the volume dilution effect, nonspecific competitors that sequester the target, and chemically modified nucleic acid libraries, as has been demonstrated with the bioreceptors known as slow off-rate modified aptamers (SOMAmers) (*25*). Although such strategies have proven successful, they have two drawbacks. First, they require the immobilization of the target on a solid surface such as a microbead, which can impair binding due to the masking of target functional groups and steric hindrance. Second, the selection strategies and reagents associated with SOMAmer generation, which are currently the only known means of obtaining slow k_{off} aptamers, are not accessible to the public, limiting their broad utilization.

RESULTS

Working principle of NA-SELEX

We present here a new rational in vitro selection method that applies kinetic selection pressure via the use of nucleases to directly isolate aptamers with slow k_{off} from randomized oligonucleotide libraries in solution. Our nuclease-assisted SELEX (NA-SELEX) method uses a structured 73-nucleotide (nt) DNA library featuring an 8–base pair (bp) stem and a 30-nt randomized loop that serves as the putative target-binding domain, flanked by constant regions containing polymerase chain reaction (PCR) primer-binding sites (Fig. 1A, library). The nuclease used here, flap endonuclease 1 (FEN1), is a high-fidelity structure–specific enzyme that rapidly cleaves 5′ overhanging flaps on DNA substrates containing upstream and downstream double-stranded regions (*26*). To enable FEN1-based separation of binding sequences, the library is hybridized with a 40-nt complementary DNA (NA-cDNA) (Fig. 1A, NA-cDNA), which forms a 15-bp double-stranded region near the 5′ terminus of the library molecule. The resulting complex offers an ideal substrate for FEN1, containing upstream and downstream duplex DNA regions with a protruding 8-nt 5′ library flap and a 3′ single-nucleotide cDNA overhang (Fig. 1B, library-cDNA complex). Library oligonucleotides that do not bind the target retain their double-flap structure and are efficiently cleaved by FEN1, which trims away 9 nt from the 5′ end (Fig. 1B, nontarget binders). In contrast, aptamers that bind the target and undergo concomitant displacement from NA-cDNA convert from a double-flap to a stem-loop structure that is unrecognizable by FEN1, and thus remain intact (Fig. 1B, aptamer-target complex). To select

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aptamers with slow k_{off}, the library is digested with FEN1 for an extended duration. Aptamers that stay bound to their target for long periods of time due to a slow k_{off} (i.e., long residence times, defined as $1/k_{off}$) are able to survive the digestion trial to a greater extent, whereas library sequences with a more rapid k_{off} release the target and readily rebind the cDNA to form a double-flap structure, which is rapidly cleaved by FEN1 (Fig. 1C). Following digestion, the intact and cleaved library strands are purified by polyacrylamide gel electrophoresis (PAGE), and the intact binders are then PCR-amplified to generate an enriched pool that is used to perform another round of selection. Once the pool demonstrates a notably increased level of resistance to FEN1 digestion, it is subjected to high-throughput sequencing (HTS) to identify the enriched aptamers (Fig. 1C).

NA-SELEX for cocaine aptamers

We used cocaine as the first demonstration selection target for NA-SELEX, given that the existing aptamer has a mediocre affinity (K_d ~ 5 μM) (*27*, *28*) and poor specificity (*29*–*31*), hence the need for an aptamer with improved binding properties. Our initial attempts with NA-SELEX proved unsuccessful, as after several rounds of selection, we observed that enriched pools resisted FEN1 digestion regardless of whether the target was absent or present. HTS analysis of these pools revealed that nontarget-binding sequences were being enriched that resisted FEN1 digestion via various mechanisms such as mutations in the cDNA hybridization site and structural misfolding (figs. S1 to S3). To overcome these issues, we first performed conventional library-immobilized SELEX (LI-SELEX) (*32*, *33*) to pre-enrich target-binding aptamers, and then used NA-SELEX with the enriched pool to specifically select aptamers with slow k_{off}. LI-SELEX entails hybridizing the library with a 15-nt biotinylated cDNA (LI-cDNA) and immobilizing the library-cDNA complex onto streptavidin-coupled agarose resin (see table S1 for sequences). Library strands that undergo displacement from the cDNA upon the addition of the target are eluted, captured, and PCR-amplified, and the resulting amplicons are used for another round of selection. We performed eight rounds of LI-SELEX to pre-enrich cocaine-binding sequences (detailed selection conditions in table S2). In the first round, we used 1 nmol of the library (~10^{14} unique sequences) and 100 μM cocaine and observed 0.5% pool elution. In the second round, we initiated counter-SELEX (*34*) to remove binders to interferents, including molecules known to bind three-way junction–structured aptamers (which typically have poor specificity) (*35*, *36*) and structurally similar interferents relevant to drug screening (see table S2 for list of interferents). For rounds 2 to 7, ~0.8% of the library was eluted by the target on average. In round 8, target-induced pool elution tripled (2.4%; fig. S4), indicating enrichment of target-binding sequences, and we, therefore, used this enriched pool to perform NA-SELEX.

We then performed three rounds of NA-SELEX at room temperature to enrich slow k_{off} aptamers (see table S3 for conditions). The overall scheme of NA-SELEX is shown in Fig. 2. Specifically, the round 8 enriched pool was hybridized with LI-cDNA, immobilized onto streptavidin beads (Fig. 2, A and B), and washed repeatedly with buffer to remove library strands that were unable to bind strongly to LI-cDNA (we refer to this as negative selection) (Fig. 2C). This was imperative, as sequences incapable of cDNA hybridization could resist FEN1 digestion without being able to bind the target, thereby carrying over to subsequent rounds. Then, an abbreviated counter-SELEX procedure was performed to remove interferent-binding sequences (Fig. 2D). The remaining library was released from the agarose resin using sodium hydroxide, and the solution was neutralized and desalted (Fig. 2E). In general, we observed that approximately 60 to 70% of the pool was recovered after negative selection and counter-SELEX in each round (fig. S5, red box). The

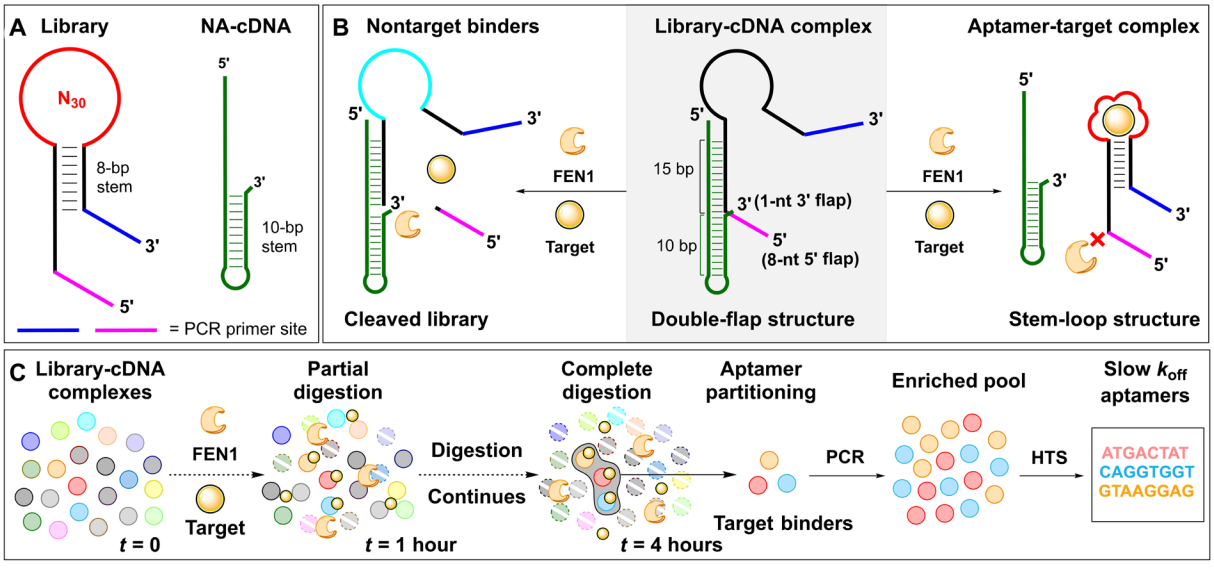

Fig. 1. The working principle of NA-SELEX for isolating aptamers with slow off-rates (k_{off}). (**A**) The 73-nt library molecule and 40-nt NA-cDNA were used for the selection process. (**B**) The library and NA-cDNA hybridize to form a complex containing downstream and upstream double-stranded DNA regions as well as an 8-nt 5′ flap and single-nucleotide 3′ overhang (middle). In the absence of the target, the 5′ flap is rapidly cleaved by FEN1 (left); in the presence of the target, the library strand dissociates from the cDNA and reverts to a stem-loop structure that is unrecognizable by FEN1 (right). (**C**) Library-cDNA complexes are digested with FEN1 in the presence of the target for several hours. Over time, sequences unable to bind the target are digested, while those that stay bound to the target for long durations survive. These binders are PCR-amplified, and the process is iterated again several times, after which slow k_{off} aptamers are identified through HTS.

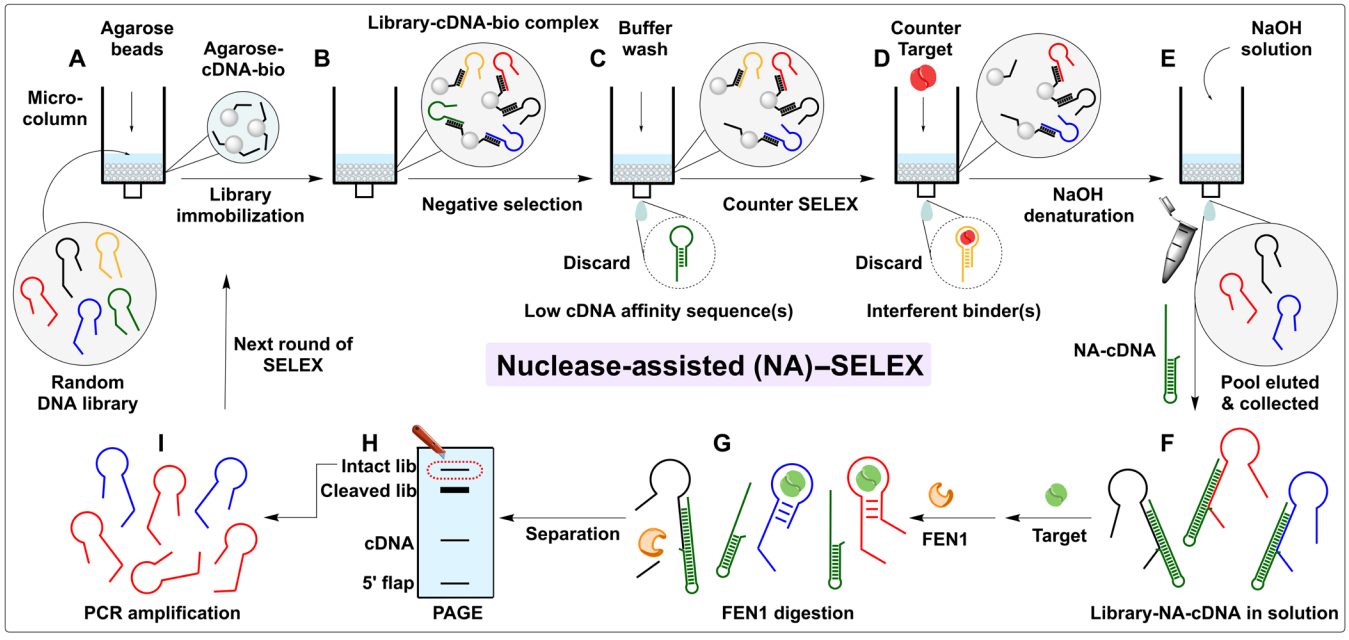

Fig. 2. The detailed procedural workflow for NA-SELEX. NA-SELEX entails (**A** and **B**) immobilization of DNA library on agarose beads, removal of (**C**) low cDNA affinity sequences via negative selection, and (**D**) interferent binders through counter-SELEX. (**E**) The remaining sequences are eluted from the agarose beads by disrupting the cDNA-library duplex with sodium hydroxide. (**F**) This library is hybridized with NA-cDNA to form library-NA-cDNA complexes with double-flap structure, which are then subjected to (**G**) target binding and FEN1 digestion, (**H**) separation of target binders (intact sequences) from nontarget binders (cleaved sequences) using polyacrylamide gel electrophoresis (PAGE), and (**I**) PCR amplification of the intact target-binding sequences.

recovered pool was then hybridized with NA-cDNA to form a flap substrate complex (Fig. 2F) and subsequently challenged with 50 μM cocaine or buffer as a negative control. The pools were digested with FEN1 for an extended period to specifically isolate slow k_{off} aptamers (Fig. 2G). After digestion, the intact target binders were separated from the cleaved sequences using PAGE and PCR-amplified for use in the next round of NA-SELEX (Fig. 2, H and I). On the basis of preliminary testing with the native random library (fig. S6), we digested the selection pools for 4 hours—a duration that was sufficient to achieve the lowest level of library retention. For the first round of NA-SELEX, 15% of the pool was retained in the absence of cocaine after digestion relative to 21% in the presence of cocaine. As a control, we determined that ~10% of the random library was retained in the same amount of time, regardless of the absence or presence of the target (Fig. 3, A and B). Elevated resistance to FEN1 digestion in the presence of cocaine indicated that the pool contained target-binding aptamers. After isolating and amplifying the selected library strands, we performed two more rounds of NA-SELEX as described above. Target-specific resistance to FEN1 digestion rose in rounds 10 and 11 to 30 and 59%, respectively, while pool retention in the absence of cocaine was 13 and 23%, respectively (Fig. 3, A and B). These results indicate that sequences were being enriched that could survive FEN1 digestion for prolonged periods of time. As a comparison, LI-SELEX was carried out for three additional rounds in parallel, with the expectation that this method would not select for slow k_{off}. During the selection, we observed increasing target elution by cocaine over rounds 9 to 11, rising from 9.5% in round 9 to 33% in round 11 (Fig. 3C), indicating that the final pool was highly enriched with cocaine-binding sequences.

HTS analysis of NA-SELEX and cocaine aptamer characterization

The round 8 to 11 LI-SELEX pools and round 9 to 11 NA-SELEX pools were then subjected to HTS to identify enriched aptamer sequences (see table S4 for a summary of HTS data). The number of unique sequences exponentially declined in the LI-SELEX pools from rounds 8 to 11, whereas we observed a linear decrease in the NA-SELEX pools (fig. S7). This suggests that different subpopulations of aptamers may have been enriched through differing selection forces in each strategy. Since both the round 11 NA- and LI-SELEX pools originated from the same round 8 LI-SELEX pool, we observed high similarity (90%) among those sequences with an abundance >0.01% in each set of pools (fig. S8). The main differences between these pools could be observed in the relative abundances and growth patterns of particular sequences (figs. S8 to S10). For example, aptamer NC76, which appeared in the final pools for both LI- and NA-SELEX, was 30-fold more abundant in the latter pool. To identify those aptamer candidates that were preferentially enriched during NA-SELEX, we analyzed these pools based on their retention factor (RF). RF is calculated on the basis of the equation $(A_T - A_C)/A_C$, where A_T and A_C are the abundance of a particular sequence in a given NA-SELEX round after digestion with or without target, respectively (Fig. 3D). Taking A_C into account minimizes the effects of PCR bias and eliminates non-binding sequences that inherently resist FEN1 digestion. Sequences with RF > 0 were presumably able to survive FEN1 digestion to a greater extent in the presence of cocaine relative to other sequences; thus sequences with relatively high RF may have slow k_{off}. In contrast, sequences with RF ≤ 0 were incapable of surviving FEN1 digestion even when the target was present, indicating they do not bind cocaine or exhibit a

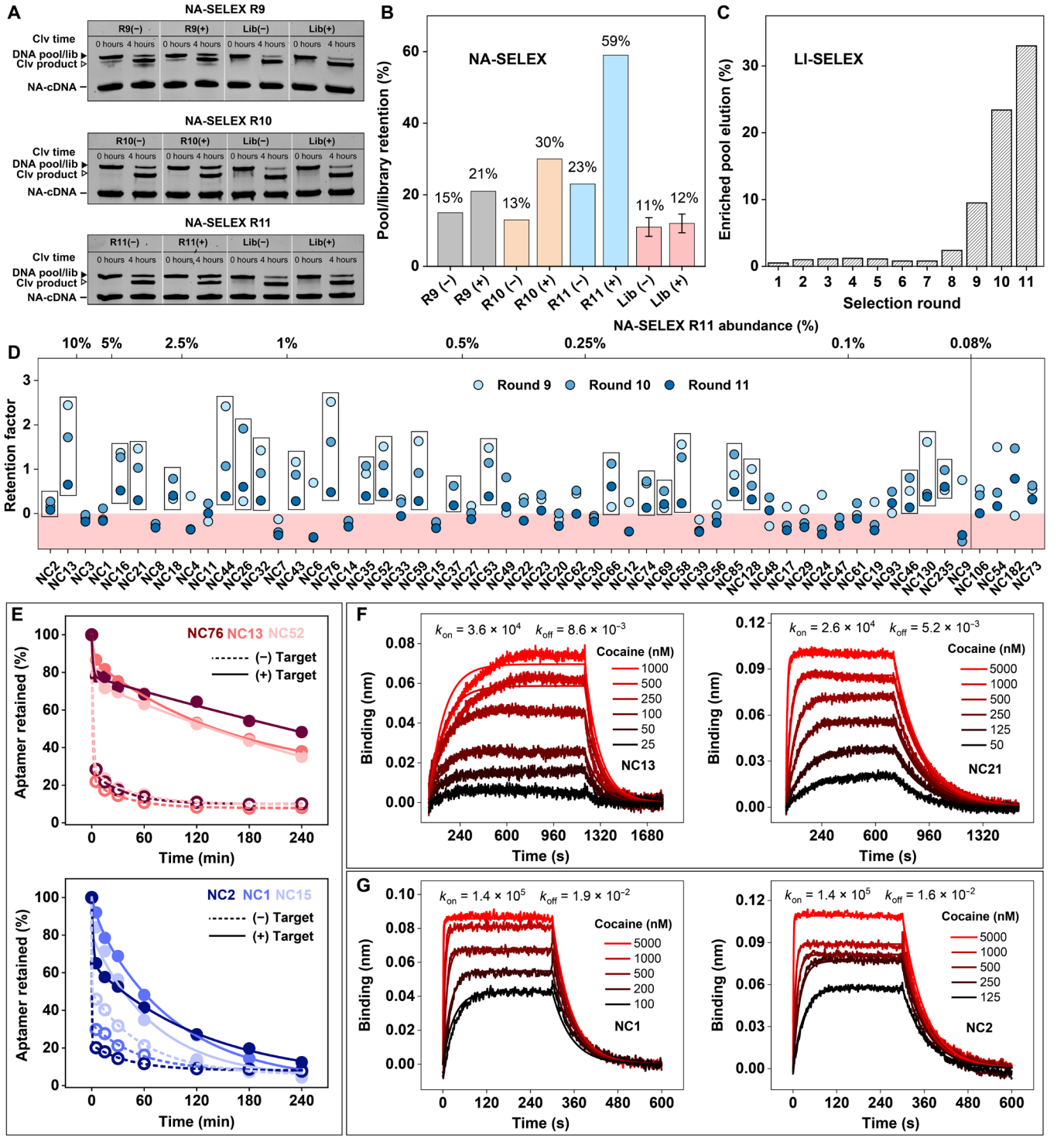

Fig. 3. Room temperature NA-SELEX yields slow off-rate aptamers for cocaine. (**A**) PAGE analysis of the round 9 to 11 (R9–11) NA-SELEX pools and the naive library undergoing digestion in the presence or absence of cocaine. (**B**) The proportion of each pool retained in the gel from (A). (**C**) Percent of the R9–11 LI-SELEX pools eluted by the target. (**D**) Retention factor (RF) for sequences with an abundance of >0.08% in round 11 of NA-SELEX. Sequences are listed in order of abundance; aptamers deemed preferentially enriched by NA-SELEX are boxed. (**E**) FEN1 digestion of individual aptamers obtained with NA-SELEX (top) or LI-SELEX (bottom) in the presence or absence of cocaine. Biolayer interferometry (BLI) data depicting binding kinetic traces for (**F**) NA-SELEX–derived sequences NC13 and NC21 and (**G**) LI-SELEX–derived sequences NC1 and NC2 at various concentrations of cocaine.

rapid k_{off}. We selected promising aptamer candidates that exhibited >0.08% abundance in the final round of selection and RF > 0 for all three NA-SELEX rounds. These 25 candidates are indicated by boxes in Fig. 3D. Notably, these aptamers also displayed the greatest growth in abundance between rounds 8 and 11 of NA-SELEX (fig. S9A) and were much more abundant (10- to 30-fold) in the round 11 NA-SELEX pool relative to the round 11 LI-SELEX pool (fig. S9B). Contrary to the marked evolution that occurred during NA-SELEX, very little changed between rounds 8 and 11 of LI-SELEX. Specifically, the most abundant sequences remained the same throughout these three rounds, and only very few experienced >4-fold enrichment (fig. S10). As a control, we also selected 19 aptamers from LI-SELEX that were either most abundant or exhibited enrichment fold >2 and abundance >0.08% in round 11. We synthesized these various aptamer candidates without primer-binding sites (tables S5 and S6) and used isothermal titration calorimetry (ITC) to determine their binding affinity for cocaine. We found no meaningful difference in the average K_d values between the two sets of aptamers (380 nM for both) or in the overall range of K_d values (22 to 2650 nM for LI-SELEX, 70 to 1100 nM for NA-SELEX) (figs. S11 to S15). This suggests that NA-SELEX—at least under the conditions performed in this trial—did not necessarily enrich higher affinity aptamers. To demonstrate that the exclusion of primer-binding sites did not affect aptamer affinity, we synthesized certain full-length sequences containing these sites (tables S7 and S8) and confirmed with ITC that there was no substantial difference in affinity with or without primer sites (fig. S16).

To better understand the differential enrichment of sequences in each selection strategy, we digested full-length aptamers hybridized with NA-cDNA in the absence or presence of cocaine (Fig. 3E, top; and figs. S17 to S19). In general, all sequences were digested to 10% within 90 min in the absence of cocaine. However, when cocaine was present, NA-SELEX–derived sequences such as NC76, NC13, and NC52 greatly resisted FEN1 digestion even after 4 hours (Fig. 3E, top). In contrast, nearly all LI-SELEX–derived sequences, such as NC1, NC2, and NC15, were completely digested after 4 hours, regardless of whether or not cocaine was present (Fig. 3E, bottom; and figs. S20 and S21). Although NA- and LI-SELEX aptamers have similar cocaine-binding affinities, the resistance of NA-SELEX aptamers to FEN1 digestion in the presence of cocaine indicates that NA-SELEX may preferentially promote the enrichment of aptamers with slower k_{off}. To further understand the binding characteristics of the aptamers enriched by both selection methods, we determined their binding kinetics using biolayer interferometry (BLI). This method monitors the binding of ligands to receptors immobilized on a biosensor surface in real time, based on changes in the interference pattern of white light that occur due to the interaction between reflected light from the sensor surface and an internal reference surface (*37*). We characterized the kinetics of aptamers that were either highly abundant or had a high RF in NA-SELEX or enrichment fold in LI-SELEX. Aptamers were labeled with biotin at their 5′ terminus and immobilized onto streptavidin-coated biosensors (see table S9 for sequences). Aptamers that were preferentially enriched by NA-SELEX, such as NC13 and NC21 (Fig. 3F and fig. S22), had an average k_{off} of ~6.2×10^{-3} s^{-1} (residence time = 160 s) with k_{on} on the order of ~10^4 M^{-1} s^{-1}. In contrast, highly abundant aptamers isolated via LI-SELEX, including NC1 and NC2, generally displayed k_{on} of ~10^5 M^{-1} s^{-1} and average k_{off} of 1.8×10^{-2} s^{-1} (residence time = 56 s) (Fig. 3G and fig. S22). The binding rate constants of these LI-SELEX sequences are similar to those of other small-molecule aptamers isolated through conventional SELEX methodologies (*38*–*40*). These results together indicate that nuclease-based selection selectively enriches aptamers that have modestly (~3-fold) slower off-rates and longer residence times. The fact that the K_d values obtained via BLI and ITC were highly concordant (tables S10 and S11) indicates that the determined kinetic parameters are robust.

NA-SELEX for cocaine at physiological temperature

On the basis of these results, we hypothesized that increasing the stringency of NA-SELEX could lead to aptamers with even slower off-rates and perhaps higher affinity. To this end, we performed NA-SELEX at physiological temperature (37°C) rather than room temperature, which would increase the catalytic activity and digestion rate of FEN1, making it more difficult for low-affinity or rapid-k_{off} aptamers to survive selection. This selection approach could also yield aptamers that bind with high affinity to targets at physiological temperature, which is valuable for applications such as in vivo bioimaging, therapeutics, drug delivery, and in vivo real-time sensing. To test this, we subjected the round 8 LI-SELEX pool to FEN1 digestion at 37°C in the presence of 50 μM cocaine or buffer. On the basis of a preliminary experiment with the naive library, the FEN1 digestion rate was much more rapid at this elevated temperature (fig. S23), and we therefore reduced the digestion time during selection to 1.5 hours. As in the previous trial of NA-SELEX, we performed negative- and counterselection at the beginning of each round to remove sequences incapable of binding the cDNA and those that bound to interferents, respectively (fig. S24). Full conditions for this NA-SELEX experiment at 37°C are shown in table S12.

The round 9 pool displayed only marginally higher resistance to FEN1 digestion in the presence of cocaine (7%) versus the absence (6%) (Fig. 4A). In the following round, target-specific pool retention increased (6.6% versus 4%) (fig. S25), and by round 11, more than twice as much DNA was retained in the presence of cocaine (7.8% versus 3.5% without) (Fig. 4, A and B), demonstrating that target-binding sequences were being enriched that increasingly resisted FEN1 digestion. We subjected the resulting NA-SELEX pools to sequencing and analyzed the resulting dataset. Pool diversity decreased from 20% unique sequences in the round 8 starting pool to <10% after three rounds of NA-SELEX (fig. S26). Notably, the sequence diversity of the final pool was greater than for the room-temperature trial. Several highly enriched sequences from the RT NA-SELEX trial were also quite abundant in the final round of 37°C NA-SELEX, such as NC76. However, nine newly identified sequences were also highly enriched, including NC1947, NC314, NC1174, NC29264, NCA, NCB, NC950, NC21357, and NC358 (Fig. 4B). NC29264 was not detected at all in the round 8 LI-SELEX pool. All of these sequences had an abundance greater than 0.08% and displayed relatively high RF values ranging from 1 to 10—much greater than the RF range of aptamers identified from room temperature selection (0.2 to 3). Notably, NC1947 was enriched by more than 1000-fold between the first and final rounds of NA-SELEX, while NC29264 and NCA were enriched by 500-fold (fig. S27). In addition, certain aptamers in this round 11 pool were far more abundant relative to selection at room temperature with LI- and NA-SELEX, such as NC1947 and NCA, which were at least 100- to 300-fold more abundant (fig. S28). This indicates that only certain aptamers can survive prolonged FEN1 digestion at elevated temperatures.

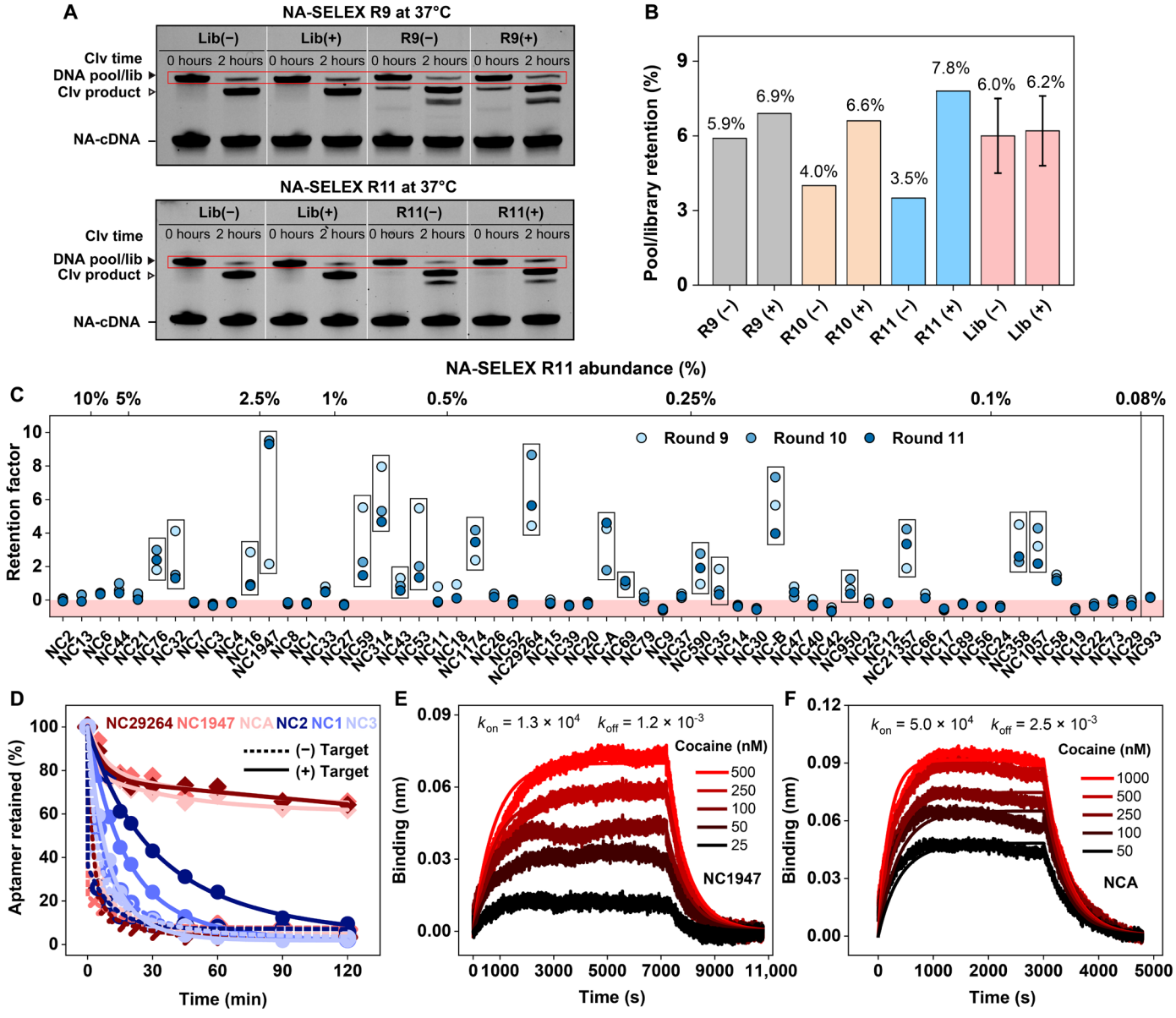

Fig. 4. Performing NA-SELEX at 37°C to yield slow-off rate aptamers that bind cocaine under physiological conditions. (**A**) PAGE analysis of the digestion of various NA-SELEX pools and the naive library with or without cocaine. (**B**) The proportion of round 9 to 11 pools retained after 2 hours in the gel is shown in (A) and fig. S25. (**C**) RFs for sequences with abundance >0.08% in the final round of NA-SELEX. Sequences are listed in order of abundance. Aptamers deemed to be preferentially enriched by NA-SELEX are boxed. (**D**) FEN1 digestion of individual aptamers preferentially enriched by NA-SELEX (red) or LI-SELEX (blue) in the absence or presence of cocaine. (**E** and **F**) BLI binding kinetics traces for (E) NC1947 and (F) NCA at various concentrations of cocaine.

Characterization of cocaine aptamers isolated at 37°C

We synthesized and characterized the target-binding affinity of 19 different primer-truncated aptamers based on abundance >0.08% and RF >1 for all three selection rounds at 37°C (Fig. 4C) using ITC (figs. S29 and S30 and table S13). Of these aptamers, 10 were already identified in the room temperature NA-SELEX trial and the other 9 were newly identified in NA-SELEX performed at 37°C. Several aptamers exhibited K_d values in the range of 170 to 2500 nM at 37°C, with a median K_d of 581 nM. This is impressive, considering that most small-molecule–binding aptamers typically display much weaker affinities at physiological temperature. For instance, the previously reported cocaine aptamer MNS4.1 (*27*) has a K_d of 49 μM at 37°C under the same buffer conditions (fig. S31), which is ~300-fold poorer than our highest-affinity aptamer, NC1947 (K_d = 170 nM). The new aptamers identified here would therefore be well suited to detect cocaine under physiologically relevant conditions. We hypothesized that the affinity of these aptamers would be even higher at room temperature. ITC confirmed that these aptamers generally had two- to fivefold greater affinity (fig. S32) with the exception of NCB and NC21357, for which cocaine binding is entropy-driven at both room temperature and 37°C.

We next examined the motifs common to NCA and related sequences including NC1947, NCB, and NC29264 since these displayed strong preferential enrichment during NA-SELEX at 37°C. Using FASTAptamer (*41*) and WebLogo (*42*), we identified conserved motifs for this family of sequences (fig. S33). Members of this family are relatively G-rich; for instance, 50% of the nucleobases in NCA are Gs. Conserved regions in this family include G-repeats interspersed with either other conserved elements (e.g., ACA at the 5′ end) or regions of low sequence conservation. To assess the importance of these G nucleobases, we designed five different point mutants of NCA wherein we converted G to T (table S14) and characterized their affinity for cocaine with ITC. None of these mutants bound to cocaine (fig. S33), indicating that these G bases are highly important for target binding. We next assessed the specificity of five of our cocaine aptamers to a variety of other small molecules using an exonuclease digestion assay based on the enzymes T5 exonuclease and exonuclease I (Exo I) (*36*). Notably, highly enriched aptamers including NCA, NCB, NC1947, NC29264, and NC21357 demonstrated excellent specificity, with <10% cross-reactivity to 34 different interferents at 100-fold higher concentrations (500 μM) relative to cocaine (certain interferent was used at <500 μM due to solubility limitations; see Materials and Methods) (fig. S34). In stark contrast, MNS4.1 cross-reacted to numerous unrelated molecules including methylenedioxypyrovalerone (MDPV) benzocaine, lidocaine, diphenhydramine, procaine, caffeine, serotonin, dopamine, and quinine.

We next performed FEN1 digestion at 37°C with full-length versions of these aptamers (including primer sites; sequences shown in table S15) in the presence of NA-cDNA with or without cocaine. All aptamers were rapidly digested by FEN1 in the absence of cocaine, but such digestion was inhibited to varying degrees when cocaine was present (Fig. 4D and figs. S35 to S37). NCA, NC1947, NC314, NC29264, and NC1174 resisted FEN1 digestion to the greatest extent in the presence of cocaine, while NCB, NC21357, NC358, NC32, NC16, NC35, and NC950 displayed only moderate inhibition. As a control, we also performed FEN1 digestion at 37°C with NC1, NC2, NC3, and NC195 which were highly abundant or enriched in the round 11 LI-SELEX pool. These sequences were rapidly digested regardless of whether cocaine was present or not (Fig. 4D and fig. S38), which explains why they were not enriched during NA-SELEX. Last, we assessed the binding kinetics of three of our NA-SELEX–enriched aptamers using BLI (Fig. 4, E and F, and fig. S39). The highly enriched aptamers NC1947 and NCA exhibited a k_{off} of 1.2×10^{-3} s^{-1} and 2.5×10^{-3} s^{-1} and a k_{on} of 1.2×10^{4} M^{-1} s^{-1} and 5.0×10^{4} M^{-1} s^{-1}, respectively. These off-rates were 10-fold lower than those of the highly abundant sequences obtained with LI-SELEX and 5-fold lower than the aptamers enriched in the room temperature NA-SELEX trial (table S16).

As a head-to-head comparison, we determined the binding kinetics of the original cocaine aptamer, MNS4.1, using BLI and found that it had a k_{off} of 2×10^{-2} s^{-1} (fig. S40), which is approximately 10-fold faster than that of NC1947. Our new cocaine aptamers have at least 10-fold slower dissociation kinetics than previously reported in vitro–selected small-molecule–binding aptamers, which have an average k_{off} of 3×10^{-2} s^{-1} with a median of 2×10^{-2} s^{-1} (table S17) and are 5-fold slower than the k_{off} of one the highest-affinity in vitro–selected small-molecule–binding aptamers reported to date [for piperaquine, $k_{off} = 5 \times 10^{-3}$ s^{-1}; (*43*)]. Our cocaine aptamers also have k_{off} on par with high-affinity riboswitches, or naturally occurring RNA aptamers, with only the lengthier, more informationally complex thiamine pyrophosphate riboswitch ($k_{off} = 2 \times 10^{-4}$ s^{-1}) and cyclic diguanosine 5′-monophosphate riboswitch ($k_{off} = 2 \times 10^{-5}$ s^{-1}) having a slower k_{off} for their targets (*39*) than NC1947. This implies that, at least for small-molecule targets, NA-SELEX can yield aptamers with k_{off} values similar to oligonucleotide receptors optimized by nature. We believe that our capability to identify slow off-rate aptamers stems from performing NA-SELEX at 37°C, which increases the catalytic activity of FEN1, and thus the threshold residence time (and hence off-rate) required to survive digestion, such that only aptamers with slow off-rates get enriched. Despite their relatively slow on-rates, NC1947 and NCA were both successfully enriched through NA-SELEX; this is most likely because of the 30 min library-target incubation period, such that these aptamers were completely bound just before the start of digestion. In future iterations of NA-SELEX, decreasing this incubation time could perhaps promote the enrichment of slow off-rate aptamers with more rapid on-rates, potentially leading to even higher affinity aptamers than those found here. These BLI data also explain why NC1947 and NCA were not enriched through conventional selection—in LI-SELEX, aptamers are eluted under nonequilibrium conditions within no more than 10 min (*32*), while these aptamers require 30 to 60 min to reach target-binding equilibrium.

Demonstrating generality of NA-SELEX with a protein target

To demonstrate that NA-SELEX could be generalized to other targets, we performed NA-SELEX to isolate DNA aptamers that bind to the serine protease thrombin. A variety of thrombin aptamers have been previously reported in the literature (*44*, *45*), giving us the opportunity to determine whether NA-SELEX could produce aptamers with better affinity and binding kinetics. We first performed five rounds of conventional filtration–based SELEX with nitrocellulose membranes (*23*) to pre-enrich thrombin-binding pools. Pool retention in the presence of thrombin consistently increased from 1% in round 1 to 14% in round 5, indicating enrichment of thrombin-binding aptamers (Fig. 5A). We then performed three rounds of NA-SELEX at 37°C as described above (Fig. 5B; selection conditions shown in table S18). In the first round, after performing an initial negative selection step (fig. S41), we digested the resulting pool hybridized to NA-cDNA with FEN1 with or without 1 μM thrombin at 37°C for 1.5 hours. Pool retention in the presence of thrombin was 30%, roughly double than in the absence of target (16%) (Fig. 5C). In contrast, we observed no difference in pool retention whether we digested the naive library with or without thrombin. In the next round, we digested the pool for a longer period (2 hours) to select specifically for slow-off rate aptamers that could survive prolonged digestion and observed 25% pool retention with thrombin relative to 14% without. In the final round, we increased selection stringency even further by performing digestion for 3 hours, obtaining pool retention of 23% with thrombin, similar to the previous round, but with a reduced background of 7% without thrombin. We terminated the selection after this round.

We then subjected all eight thrombin SELEX pools to HTS to identify aptamer sequences. The number of reads obtained for each pool is shown in table S19. Unlike NA-SELEX for cocaine, no particular aptamer dominated the final round pool in terms of abundance—the highest-ranking aptamer had an abundance of 0.0037%. The sequence diversity of the pools barely decreased from round 1 to round 7, with an average of 40 ± 2% unique sequences.

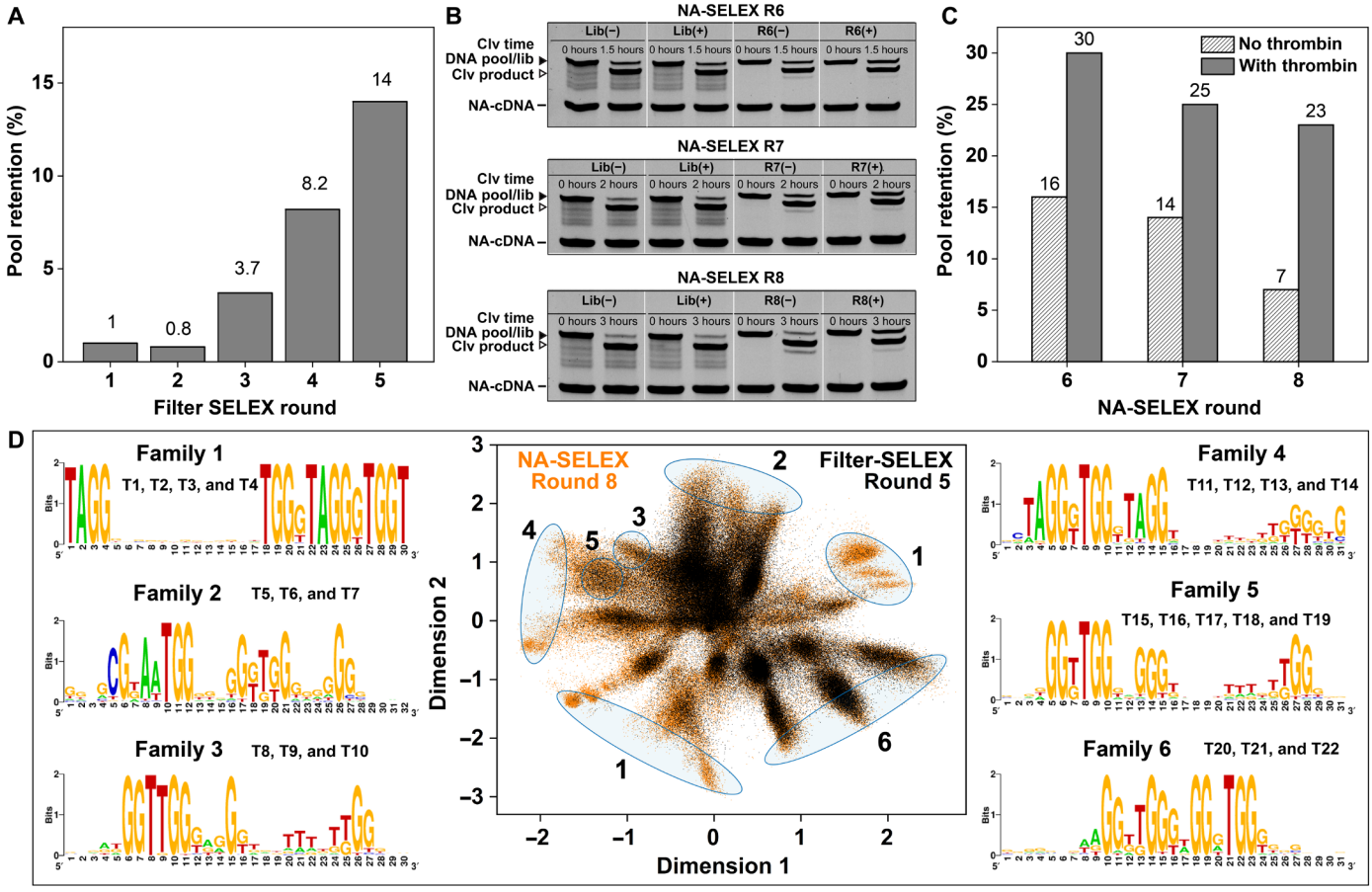

Fig. 5. NA-SELEX can generate slow off-rate aptamers for protein targets. (**A**) Pool retention for each round of filter-SELEX to pre-enrich binders to thrombin. (**B**) PAGE analysis of the digestion of various NA-SELEX pools and the naive library with or without thrombin. (**C**) The proportion of round 6 to 8 pools retained after digestion in the gel is shown in (B). (**D**) Identification of sequence families in the round 5 filter-SELEX pool (black) and the round 8 NA-SELEX pool (orange) using RaptGen. The two-dimensional plot shows the latent space produced, where each dot represents one unique sequence. Clustered sequences are related and contain similar motifs. From this analysis, we identified six sequence families. The sequence logos for these families (generated by WebLogo) contain several conserved motifs and nonconsensus regions.

Unexpectedly, the proportion of unique sequences rose in round 8 to 75%, which indicates a notable change in the composition of sequences in the pool. Thus, the pools did not converge to a handful of highly abundant sequences, as is usually observed for SELEX. To identify aptamer candidates for further characterization, we therefore analyzed the population dynamics of aptamer families instead. We used the bioinformatics software RaptGen (*46*), which uses variational autoencoders to map aptamers sequences in two-dimensional space, where sequences with similar motifs form clusters in the latent space. The round 8 pool was used as a training set to build an encoder and decoder pair. After this initial training, the model was applied to sort sequences in every SELEX round to visualize the evolution of sequence families (fig. S42). In the round 8 pool, we observed several different clusters of sequences. To determine the representative motif of each cluster, we used Clustal Omega (*47*) to align sequences and then WebLogo (*42*) to visualize each motif. We identified six unique motif families (Fig. 5D). Notably, all families contained relatively short (10 to 20 nt in length) G-rich motifs, suggesting that they most likely contain G-quadruplex structures, similar to previously reported thrombin aptamers. For instance, sequences in family 1 all contained a highly conserved 4-base TAGG motif at their 5′ end, with another 13-base motif at the 3′ end containing three GG repeats; both motifs were linked by a 13-nt region in the middle of the random domain with almost no consensus. We identified 290 sequences in the round 8 pool containing the original 15-nt thrombin aptamer sequence reported by Bock *et al.* (*44*), but none of these were members of any of the six families. In contrast, we were not able to find the thrombin aptamer reported by Tasset *et al.* (*45*) in our SELEX pools. Having identified these different aptamer families, we then assessed their enrichment throughout SELEX (fig. S42). We observed that in the filter-SELEX rounds (rounds 1 to 5), family 6 experienced the largest growth, with families 3 and 5 undergoing modest growth. From the initiation of NA-SELEX in round 6 until the final round of NA-SELEX, we observed that families 1, 2, and 4 began to gain many more members. By overlapping the sequence space in the final rounds of filter-SELEX and NA-SELEX (rounds 5 and 8), we could readily visualize that families 1, 2, and 4 were preferentially enriched by NA-SELEX, while family 6, which was enriched during filter-SELEX, primarily remained static in abundance (Fig. 5D).

Characterization of thrombin aptamers

On the basis of our analysis of the HTS data, we chose several aptamer candidates from each family (24 in total; table S20) for further affinity characterization. First, we used our T5/Exo I exonuclease digestion assay to determine the relative affinity of each aptamer for thrombin. As a point of comparison, we also included the high-affinity DNA aptamer for thrombin isolated by Tasset *et al.* [$K_d = 0.5$ nM; (*45*)] in this assay. We first digested the aptamers with or without 100 or 500 nM thrombin at room temperature and determined that most aptamers displayed target-specific resistance to digestion, with 90% of sequences having similar resistance values as the Tasset aptamer (Fig. 6A). Underperforming aptamers included those from family 3 (T9) and family 6 (T20, T21, and T22). We then performed the same assay with a subset of these aptamers to assess thrombin affinity at 37°C and observed that several of them, including the Tasset aptamer, displayed lower resistance values relative to those obtained at room temperature, which indicates that target affinity weakens at increased temperatures (Fig. 6B). Two of these aptamers, T15 and T23, had greatly diminished affinity. To evaluate the specificity of these new thrombin aptamers, we performed the exonuclease digestion assay with the subset of aptamers having the greatest relative affinity for thrombin. Impressively, none of the thrombin aptamers displayed meaningful cross-reactivity to 500 nM human serum albumin, streptavidin, or human factor Xa, another serine protease (Fig. 6C). These data indicated that NA-SELEX is capable of identifying specific binders to a protein target.

Last, we determined the binding affinity and kinetics of the best-performing thrombin aptamers (table S21) at 37°C using BLI. The K_d of the new thrombin aptamers generally ranged between 0.7 and 30 nM (Fig. 6D, fig. S43, and table S22). Notably, NA-SELEX–enriched aptamers T3, T4, T7, and T13 had affinities superior to the Bock and Tasset aptamers by one or two orders of magnitude. Specifically, the Bock and Tasset aptamers had K_ds of 18.0 and 4.7 nM, respectively, while T3, T4, T7, and T13 had K_ds of 1.7, 0.9, 0.8, and 1.8 nM at 37°C. This indicated that NA-SELEX can produce aptamers with improved affinity relative to those generated through conventional means. This is also supported by the fact that the family 6 aptamers, which were enriched early on during the filter-SELEX rounds and had relatively low resistance values, had the poorest affinity of all aptamers tested (for example, T22 K_d = 18 nM). Other aptamers also displayed modest improvement relative to previously reported thrombin aptamers, with K_d ranging between 2 and 10 nM at 37°C. In addition, we also observed that while the NA-SELEX thrombin aptamers identified here had similar on-rates to the Bock and Tasset aptamers ($k_{on} \sim 10^6\ M^{-1}\ s^{-1}$), they had notably slower off-rates (table S22). For instance, T3, T4, T7, and T13 have respective k_{off}

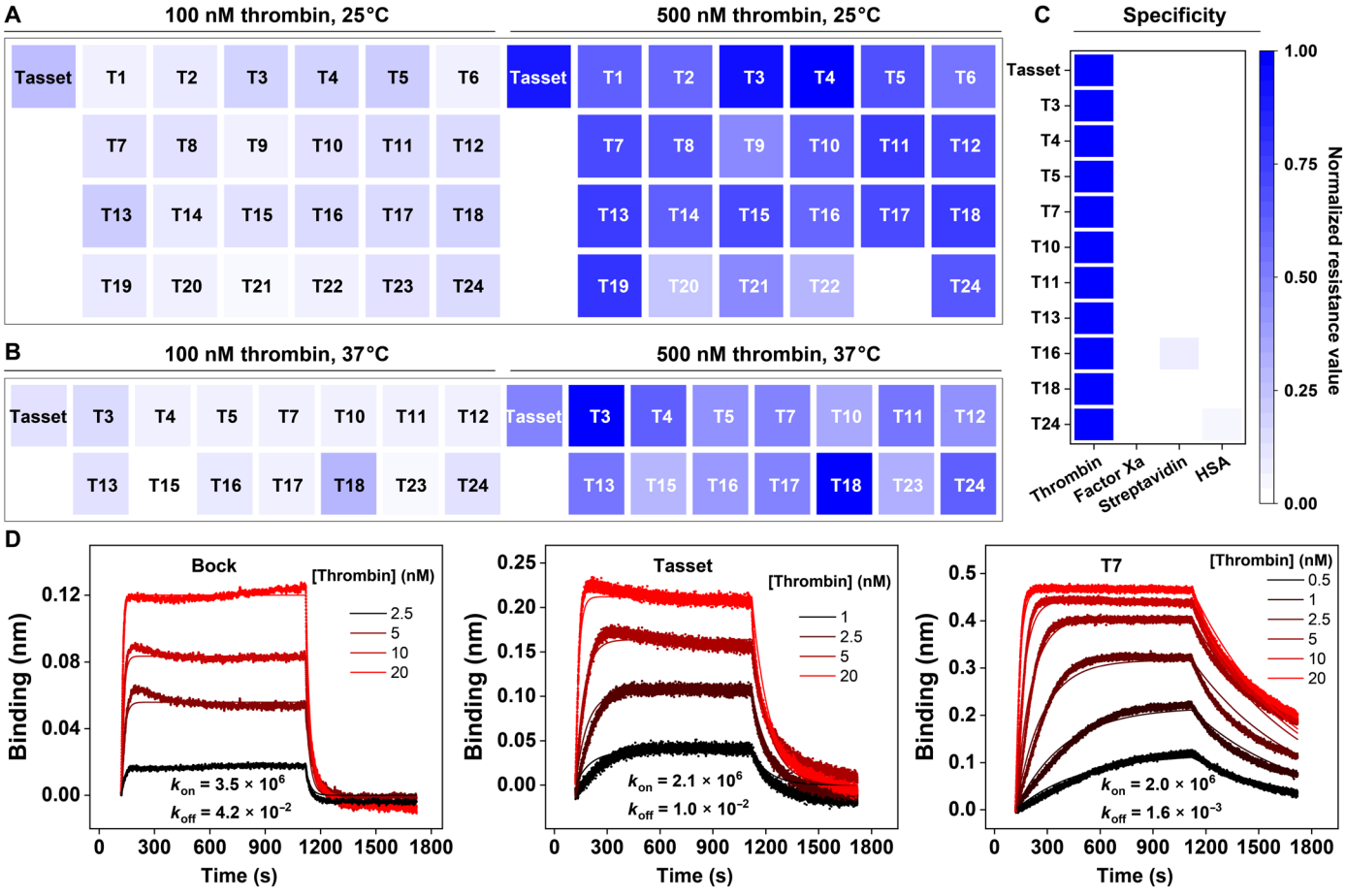

Fig. 6. Affinity and kinetic characterization reveal that thrombin-binding aptamers found via NA-SELEX have high affinity and specificity as well as slow ligand dissociation rates. Results for the exonuclease digestion assay are represented as heatmaps of thrombin affinity at (**A**) room temperature and (**B**) 37°C. Heatmaps of aptamer specificity are shown in (**C**). (**D**) BLI data to determine the binding kinetics of the Bock aptamer, Tasset aptamer, and NA-SELEX aptamer T7.

values of 3.2×10^{-3} s^{-1}, 3.4×10^{-3} s^{-1}, 1.6×10^{-3} s^{-1}, and 4.2×10^{-3} s^{-1}, an order of magnitude lower than the Bock and Tasset aptamers ($k_{off} = 4.0 \times 10^{-2}$ s^{-1} and 1.0×10^{-2} s^{-1}, respectively). These aptamers with slow off-rates were most likely enriched because of the kinetic pressure of NA-SELEX, which selects for sequences that have long residence times—in the case of T7, ~10 min. In contrast, aptamers with mediocre off-rates, such as T22 ($k_{off} = 2.6 \times 10^{-2}$ s^{-1}), did not survive the NA-SELEX process due to their short residence time (~30 s), which greatly increased their susceptibility to degradation by FEN1. These results therefore definitively demonstrate that NA-SELEX can yield slow-off rate aptamers for multiple categories of target.

DISCUSSION

We have developed a rational nuclease-based selection strategy for isolating aptamers with high affinity and specificity as well as slow off-rates. The method is relatively straightforward and is readily accessible to those with the capabilities to perform conventional SELEX. NA-SELEX also provides a new route to aptamers that function at physiological temperatures, which is currently difficult—especially for small-molecule targets. Here, we have demonstrated the capabilities of NA-SELEX with the small-molecule drug cocaine and the protein thrombin. On the basis of these successes, we foresee that this methodology should be broadly applicable. We believe that the most promising application of NA-SELEX will ultimately be for the isolation of slow off-rate aptamers for protein and cell targets. Such aptamers will be of great use for the development of nanotechnological devices (*48*–*50*), molecular imaging (*51*), drug delivery (*52*), and therapeutics (*53*), wherein long ligand-receptor residence times are crucial for successful outcomes. Last, given that only the peripheral scaffold regions of library molecules and cDNA need to consist of natural nucleic acids, we foresee that NA-SELEX could also be adapted for use with chemically modified libraries (*54*) to isolate aptamers with further improvements in binding properties as well as nuclease resistance, which will be crucial for biological applications.

MATERIALS AND METHODS

Oligonucleotides

All DNA oligonucleotides were purchased from Integrated DNA Technologies. The complementary DNA (NA-cDNA) and HTS primers were purified by the manufacturer via PAGE. The library used for cocaine selections was purified by PAGE, while the library used for thrombin selections was purified by high-performance liquid chromatography (HPLC). Primers for in vitro selection and biotinylated oligonucleotides were purchased as HPLC-purified. All other oligonucleotides were acquired as standard desalt quality. The oligonucleotides were dissolved in molecular biology–grade water and their concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

Reagents

Molecular biology–grade water was purchased from Corning. Tris base, Tris-HCl, sodium chloride, magnesium chloride solution, potassium chloride, Triton X-100, lidocaine HCl, diphenhydramine HCl, procaine HCl, benzocaine, ibuprofen sodium, acetaminophen, caffeine, quinine hemisulfate hydrate, serotonin HCl, dopamine HCl, biocytin, bovine serum albumin (BSA), 3-kDa (0.5-ml capacity) and 10-kDa molecular weight cutoff filters (4-ml capacity), and Whatman Protran nitrocellulose membranes (BA85: pore size, 0.45 μm; diameter, 25 mm) were purchased from Sigma-Aldrich. Levamisole HCl was purchased from MP Biomedicals. Scopolamine hydrobromide trihydrate was purchased from Acros Organics. Recombinant human FEN1, cocaine HCl, (−)-nicotine, mephedrone HCl, MDPV HCl, methylphenidate HCl, fentanyl HCl, (+)-methamphetamine HCl, methylenedioxymethamphetamine HCl, morphine sulfate, oxycodone HCl, fluoxetine HCl, and methadone HCl were purchased from Cayman Chemicals. Tris-EDTA (TE) solution (pH 8.0, 1×), formamide, phenol-chloroform-isoamyl alcohol solution (Invitrogen), urea, heavy phase lock gel tubes (Quantabio), and dithiothreitol (DTT; Roche) were purchased from Thermo Fisher Scientific. Microgravity columns (800 μl) were purchased from Bio-Rad. Streptavidin-coated agarose resin (capacity: 1 to 3 mg of biotinylated BSA/ml of resin) and SYBR Gold were purchased from Thermo Fisher Scientific. Human α-thrombin and human factor Xa were purchased from Prolytix. Streptavidin was purchased from Thermo Fisher Scientific. Native human serum albumin was purchased from Abcam. GoTaq Hot Start Colorless Master Mix was purchased from Promega. PCR purification kits were purchased from Qiagen. Octet Streptavidin (SA) and Super Streptavidin (SSA) biosensors for BLI experiments were purchased from Sartorius.

Buffers

For the selection of cocaine aptamers, the buffer consisted of 20 mM Tris (pH 7.4), 140 mM NaCl, 4 mM KCl, and 5 mM $MgCl_2$. For cocaine NA-SELEX, the buffer for FEN1 digestion also included 0.008% (v/v) Triton X-100 and 1 mM DTT. For the selection of thrombin aptamers, the buffer consisted of 20 mM tris (pH 7.4), 140 mM NaCl, 4 mM KCl, and 1 mM $MgCl_2$. For thrombin NA-SELEX, the buffer for FEN1 digestion also included 0.01% (v/v) Triton X-100. BLI experiments for thrombin aptamers included 7.5 μM BSA in the buffer. Loading buffer (1×) for PAGE analysis contains 75% formamide (v/v), 10% glycerol (v/v), 0.125% SDS (w/v), 10 mM EDTA, and 0.02% (w/v) xylene cyanol. PAGE purification buffer (prepared as 2×) contains 7 M urea, 40% glycerol (v/v), and 0.02% (w/v) xylene cyanol.

Library-immobilized–SELEX

LI-SELEX was performed according to a previously reported protocol (*32*). Each library strand is 73 nt in length and contains an 8-bp stem, a 30-nt randomized domain, and is flanked with PCR primer sites. The library contains a docking sequence for hybridization with a 15-nt complementary DNA strand. The sequences used for SELEX are listed in table S1 and detailed selection conditions are provided in table S2. The DNA library was first hybridized to a 15-nt biotinylated complementary DNA strand (LI-cDNA15-bio) by dissolving both in cocaine selection buffer at a molar ratio of 1:5, incubating at ~95°C for 5 min in a boiling water bath, and subsequently cooling in a room temperature water bath for 20 min. In the meantime, 250 μl of streptavidin agarose resin was loaded into a microgravity column and the resin was washed five times with 250 μl of selection buffer. The library-cDNA solution was then added to the column to immobilize the library. The immobilized library was washed several times with 250-μl aliquots of a selection buffer to remove nonhybridized library molecules. Counter-SELEX was performed at this point to remove sequences that bound to interferents. The column

was then washed again several times with 250 μl of buffer. Cocaine dissolved in selection buffer was then added to the column in three 250-μl aliquots, and the eluent was collected and subjected to purification with a 3-kDa ultracentrifugation filter to remove the target and salts. Library molecules in the purified eluent were amplified by performing PCR with 1 ml of GoTaq Hot Start Colorless PCR Master Mix, 1 μM forward primer (FP), and 1 μM biotinylated reverse primer (RP-bio) with a Bio-Rad C1000 thermal cycler using the following protocol: 2 min at 95°C, 11 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 45 s, followed by 5 min at 72°C. The optimal number of amplification cycles was determined via PAGE analysis of PCR samples. Single-stranded DNA was generated from the resulting double-stranded PCR amplicons as reported previously and lastly purified with a 3-kDa filter with water. The concentration of DNA in the pool was determined using a NanoDrop2000 spectrometer.

NA-SELEX for cocaine aptamers

Each round of NA-SELEX consists of four parts and is described in detail below.

Part 1. Negative- and counterselection

NA-SELEX was initiated using the pool from round 8 of LI-SELEX. Washing (negative selection) and counter-SELEX were first performed to remove library sequences incapable of stable hybridization with the cDNA and those that bound to interferents, respectively. Detailed conditions for each round are provided in tables S3 and S12. The library pool was first hybridized with a fivefold excess of LI-cDNA15-bio in selection buffer by heating the solution in a boiling water bath for 5 min and cooling it in a room-temperature water bath for 20 min. In the meantime, 250 μl of streptavidin agarose resin was loaded in a microgravity column and washed five times with 250 μl of selection buffer. The library-cDNA solution was then added to the column to immobilize the library. The column was washed 30 times with a selection buffer to remove weakly bound library strands, and then an abbreviated counter-SELEX procedure was performed against a variety of interferents. These include group TWJ1 (300 μM each of procaine, diphenhydramine, and nicotine), group TWJ2 (300 μM each of procaine, levamisole, and benzocaine), and 300 μM fentanyl. To perform counter-SELEX, 250 μl of interferents was added to the column, and the eluents were discarded. Afterward, the column was washed 30 times with 250-μl aliquots of selection buffer. When NA-SELEX was performed at 37°C, these washes were performed with selection buffer prewarmed to 37°C in a water bath; the pH of the buffer was 7.4 at 37°C. To increase the recovery of the library from the beads, the column was washed five times with 250 μl of a selection buffer without $MgCl_2$. This was done because the efficiency of elution with sodium hydroxide is lower when Mg^{2+} is present. The column was then capped and 300 μl of 0.2 M NaOH was added to disrupt library-cDNA interaction. After 10-min incubation at room temperature, the column was uncapped and the eluent was collected, neutralized with 1 M HCl, and subsequently purified with a 3-kDa filter to remove salts. The concentration of DNA in this purified solution was determined using a NanoDrop2000 spectrometer.

Part 2. FEN1 Digestion

After negative- and counterselection, the resulting pool was then subjected to positive selection using FEN1. The reaction volume for this step is 100 μl. Two samples were prepared: the library-cDNA complex plus target, and a "background" sample containing library-cDNA complex but no target as a control. First, the pool was hybridized with a fivefold excess of NA-cDNA in the selection buffer using the heating and cooling procedure described above. Then, Triton X-100 and DTT were added to reach a final concentration of 0.008% (v/v) and 1 mM, respectively. Afterward, either buffer or cocaine (final concentration 50 μM) was added to the library-cDNA mixture, which was then incubated at 25°C or 37°C for 30 min to allow the target to bind. Before digestion, a 1-μl aliquot of each reaction solution was taken and mixed with a loading buffer for analysis with PAGE. To initiate digestion, we added FEN1 (final concentration: 0.35 U/ml) in buffer containing 20 mM Tris, 0.008% (v/v) Triton X-100, and 1 mM DTT to the samples. The digestion was allowed to proceed for 4 hours at 25°C or 1.5 hours at 37°C. Before termination of digestion, a 1-μl aliquot was collected and added to the loading buffer for PAGE analysis. To halt digestion, EDTA was added (final concentration 100 mM) and the samples were heated for 10 min at 75°C to denature FEN1. The samples were subsequently purified using a 3-kDa filter to remove EDTA, salts, and the target. The final volume of samples after purification was typically 50 to 100 μl.

Part 3. PAGE Purification

PAGE was performed to separate intact library strands from cleaved library products and NA-cDNA. Digestion samples were concentrated using a vacuum centrifuge to approximately 5 μl and mixed with an equivalent volume of 2× PAGE purification buffer. The samples were subsequently loaded in a urea-denatured 12% polyacrylamide gel. Electrophoresis was performed initially at 100 V (5 V/cm) for 30 min followed by 400 V (20 V/cm) for 90 min. The gel was consistently kept warm throughout the separation process with 0.5× tris-borate EDTA (TBE) buffer warmed to 65°C to maintain the DNA in a denatured state. Afterward, the gel was removed from the apparatus and illuminated with a 284-nm ultraviolet lamp to locate DNA bands. The intact library was excised from the gel; in cases where the intact library could not be visually identified, a rectangular incision was made above the cleaved library band. The incised gel was then crushed with a 1-ml syringe plunger and soaked in 1× TE buffer for 3 hours at 60°C in a shaking incubator to elute aptamers from the gel. Afterward, the crushed gel solution was centrifuged at 7000 rcf for 15 min and the supernatant containing DNA was removed and purified with a 0.45-μm filter. The DNA was then concentrated and purified using a 15-ml 10-kDa molecular weight cutoff filter. The final volume of the DNA solution was approximately 100 μl.

Part 4. PCR and single-strand generation

Last, the purified DNA was PCR-amplified with 1 ml of GoTaq Hot Start Colorless Master Mix, 1 μM FP, and 1 μM reverse primer (RP-bio) with a Bio-Rad C1000 thermal cycler using the following protocol: 2 min at 95°C, 11 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 45 s, followed by 5 min at 72°C. Single-stranded DNA was generated from the resulting double-stranded PCR amplicons as reported previously and lastly purified with a 3-kDa filter with water. The concentration of single-stranded DNA in the pool was determined using a NanoDrop2000 spectrometer. This pool was used for subsequent rounds of NA-SELEX.

Filter-SELEX

Aptamers binding to thrombin were pre-enriched using a previous protocol based on nitrocellulose membrane filtration (*23*). To initiate SELEX, the 30-nt stem-loop library was first dissolved in 200 μl of thrombin selection buffer, heated to 95°C for 5 min in a boiling

water bath, and subsequently cooled to room temperature for 20 min. Afterward, BSA was added to the library solution to a final concentration of 1.5 μM (0.01%). To perform negative selection, the library solution was incubated with a piece of nitrocellulose filter in a 2-ml tube for 15 min, after which the filter was discarded. The supernatant was subjected to negative selection once more, in the same manner, to thoroughly remove filter binders. The library solution was then split into 100-μl portions in two 2-ml tubes and incubated with either selection buffer (negative control) or 1 μM thrombin for 15 min. The solutions were then subjected to vacuum filtration using a nitrocellulose membrane and a Millipore vacuum filtration apparatus with a pressure of 20 cmHg. After prewashing the membrane with 5 ml of selection buffer, the library solution was added to the membrane, which was then washed again with 5 ml of buffer to remove nonbinding sequences. Then, the membrane was cut into eight pieces and placed into a 2-ml tube and incubated at 95°C for 5 min in 400 μl of urea solution [7 M urea, 3 mM EDTA, and 10 mM Tris, (pH 7.4)] to remove aptamers from the membrane. The supernatant containing aptamers was removed and kept. This extraction procedure was performed once more to maximize aptamer recovery. Then, the aptamer-containing solution was diluted twofold with purified water and subjected to phenol-chloroform-isoamyl alcohol extraction to remove proteins. The aqueous layer, containing the aptamers, was kept and further purified with water and a 10-kDa molecular weight cutoff filter. PCR was then performed as described above to amplify aptamers, followed by a single-strand DNA generation step using the resulting amplicons. This selection procedure was performed for five rounds in total.

NA-SELEX for thrombin aptamers

NA-SELEX was initiated using the pool from the fifth round of filter-SELEX. Detailed conditions for each round are provided in table S18. The library was hybridized with a fivefold excess of LI-cDNA15-bio in a selection buffer and then immobilized on streptavidin-coated agarose resin loaded in a microgravity column as described above. The column was washed 20 times with selection buffer and 10 times with selection buffer warmed to 37°C to remove weakly bound library strands. Then, the column was washed three times with a selection buffer without $MgCl_2$. The column was then treated with NaOH as explained above to remove the remaining library from the column, and the solution was subsequently pH neutralized and purified with a 3-kDa filter to remove salts. After negative selection, the resulting pool was then subjected to positive selection using FEN1. Two 100-μl samples were prepared. First, the pool was hybridized with a 2.5-fold excess of NA-cDNA in the selection buffer. Then, Triton X-100 was added to a final concentration of 0.01% (v/v). Afterward, either buffer or thrombin (final concentration: 1 μM) was added to the library-cDNA mixture, which was then incubated at 37°C for 15 min to allow the target to bind. To initiate digestion, FEN1 was added (final concentration: 0.35 U/ml) to the samples in buffer containing 20 mM Tris and 0.01% (v/v) and Triton X-100. Digestion was allowed to proceed for 1.5 hours for round 6, 2 hours for round 7, and 3 hours for round 8 at 37°C. To stop digestion, EDTA was added (final concentration: 100 mM) and the samples were heated for 10 min at 75°C. The samples were subsequently purified using phenol-chloroform-isoamyl alcohol extraction and then with water and a 3-kDa filter to remove EDTA, impurities, and salts. PAGE purification was performed as explained above to separate intact library strands from cleaved library products and NA-cDNA. Last, the purified DNA was PCR-amplified, and single-stranded DNA was generated from the resulting double-stranded PCR amplicons as described above. This pool was used for another round of NA-SELEX.

DNA sequencing

Enriched oligonucleotide pools from in vitro selection were submitted to Genewiz for Illumina-based HTS. To prepare the samples, pools were PCR-amplified using primers containing partial Illumina adapters (HTS-FP and HTS-RP) with the PCR protocol described above. PAGE was performed to confirm successful amplification. Afterward, the amplicons were purified using a PCR purification kit (Qiagen) and dissolved in 10 mM Tris buffer (pH 7.4) at a concentration of 20 ng/μl. These samples were then directly submitted to Genewiz.

Bioinformatic analysis

HTS data were received from Genewiz as fastq files of forward and reverse reads. The number of reads and unique sequences in each file can be found in tables S4 and S19. To analyze the HTS data, the reverse reads were first converted to their complement using the FASTX toolkit and were then combined with the forward reads. Subsequently, cutadapt (*55*) was used to trim 5′ and 3′ constant regions with an allowed error of 20%. Any sequences containing an "N" read or having a variable region shorter or longer than a preset cutoff were discarded. FASTAptamer (*41*) was used to count sequences and determine sequence abundance in each pool. The enrichment fold between rounds or RF between negative and positive NA-SELEX samples was determined using these abundance values. RF was determined using the equation $(A_T - A_C)/A_C$, where A_T and A_C are the abundance of a particular sequence from an NA-SELEX round digested with or without a target, respectively. Certain sequences that were absent in a pool were assigned the minimum reads per million value for that pool to facilitate the calculation of the enrichment fold. For analysis of the initial failed trials of NA-SELEX, Clustal Omega (*47*) was used to align sequences and identify motifs. For thrombin selection pools, RaptGen was used to identify aptamer motifs (*46*). The round 8 NA-SELEX pool was used to generate the model using a cutoff of six reads. The building of the model converged after 430 iterations. This model was used to represent the latent space of rounds 1 to 8 to evaluate the evolution of sequence families. Families were identified from clusters of sequences at the extremities of the latent space. Sequence logos for these families were made using WebLogo (*42*). HTS data have been uploaded to the NCBI Sequencing Read Archive.

Isothermal titration calorimetry

These experiments were performed using a Malvern Microcal iTC200 or Microcal PEAQ ITC at either 23° or 37°C. First, the aptamer (final concentration: 15 μM) was dissolved in 20 mM Tris buffer (pH 7.4), heated for 5 min at 95°C in a dry bath incubator, and cooled immediately on ice for 3 min. Then, salts were added to achieve the final composition of the selection buffer. Approximately 300 μl of aptamer solution was loaded in the cell, and cocaine (final concentration: 150 μM) was loaded in the titration pipet. After a 60-s initial delay, a 0.4-μl injection was performed followed by 19 consecutive 2-μl injections with spacing of 150 s; spacing was adjusted for some titrations to up to 300 s due to slow equilibration. Data were analyzed using the MicroCal analysis kit integrated into Origin 7 software with a one-site binding model.

FEN1 digestion

These experiments were conducted in a dry bath incubator at either 25° or 37°C with a total volume of 50 μl. First, 1 μM aptamer and 5 μM NA-cDNA1 (final concentrations) were dissolved in selection buffer, heated in a boiling water bath for 5 min, and subsequently cooled in a room temperature water bath for 20 min. Afterward, Triton X-100 and DTT were added (final concentrations: 0.008% v/v and 1 mM, respectively), followed by brief mixing. Cocaine or buffer (50 μM; as a control) was then added to the aptamer solution and allowed to incubate for 30 min to permit binding. A 1-μl sample was taken before the start of the digestion and mixed with loading buffer to determine the initial aptamer concentration via PAGE. Thereafter, FEN1 (final concentration: 0.35 U/ml) dissolved in 20 mM tris with 0.008% (v/v) Triton X-100 and 1 mM DTT was added to the aptamer solution to initiate digestion. Aliquots (1 μl) were taken periodically and mixed with 32 μl of loading buffer to quench the reaction. Samples dissolved in loading buffer were subjected to PAGE using a Bio-Rad Mini-Protean gel system. Specifically, samples were run in warm 0.5× TBE (65°C) first at 50 V for 10 min and then at 200 V for 30 to 45 min. Gels were then stained with 1× SYBR Gold for 15 min and subsequently imaged using a Bio-Rad Gel Imaging System. The relative quantity of library retained during digestion was determined by comparing the intensity of the intact aptamer band before digestion relative to the intensity of intact aptamer bands from samples taken during the digestion process. Aptamer retention was plotted against time to create a digestion time-course plot, which was fitted using Origin 2021b software with a biexponential decay equation.

Exonuclease digestion assay

These experiments were performed to assess the specificity of the cocaine- and thrombin-binding aptamers. Each aptamer was initially diluted in Tris buffer (final concentration: 20 mM) and heated to 95°C for 10 min, after which they were immediately cooled on ice. Then, BSA and salts were added to reach appropriate final concentrations [140 mM NaCl, 4 mM KCl, 5 mM $MgCl_2$ for cocaine aptamers/1 mM $MgCl_2$ for thrombin aptamers, and BSA (0.1 mg/ml)]. Subsequently, either buffer, target (final concentrations: 5 or 100 μM for cocaine and 0.1 or 0.5 μM for thrombin), or interferent [final concentrations: 500 μM for all except for THC, AB-FUBINACA, and UR-144 (5 μM); alprazolam and diazepam (50 μM); quinine (250 μM); and human factor Xa, streptavidin, and human serum albumin (0.5 μM)]. For samples containing THC, AB-FUBINACA, UR-144, alprazolam, or diazepam, 5% dimethyl sulfoxide (v/v) was included in the buffer. For cocaine aptamers, samples were incubated for 30 min at 25°C to allow aptamer–ligand binding to reach equilibrium. For thrombin aptamers, samples were incubated for 30 min at 25° or 37°C. Then, 25 μl of a mixture of T5 exonuclease (final concentrations: 0.2 U/μl at 25°C or 0.067 U/μl at 37°C) and Exo I (final concentrations: 0.015 U/μl at 25°C or 0.005 U/μl at 37°C) dissolved in buffer containing BSA was added to the sample to initiate digestion. Cocaine aptamers were digested at 25°C, and thrombin aptamers were digested at either 25° or 37°C. Time points were collected by taking 5 μl of the sample and adding to 30 μl of quenching solution [final concentrations: 10 mM tris (pH 7.4), 1× SYBR Gold, 21 mM EDTA, and 12.5% formamide] loaded in a black 384-well flat bottom microplate. The fluorescence of the samples was measured using a Tecan M1000 Pro microplate reader with an excitation wavelength of 495 nm and an emission of 537 nm. Each sample was measured 10 times and the average of these measurements was used for analysis. Resistance values were calculated using the formula $(AUC_L/AUC_0) - 1$, where AUC_L and AUC_0 are the area under the curve of the fluorescence time-course plots with and without ligand, respectively. Cross-reactivity was calculated using 100 μM cocaine or 0.5 μM thrombin as 100%.

Biolayer interferometry

The binding kinetics of the cocaine aptamers were determined using BLI with a Sartorius Octet R4 instrument at 23°C. First, Super Streptavidin biosensors (Sartorius) were immersed in selection buffer for at least 15 min to hydrate the sensors. In parallel, a 100 nM solution of 5′ biotinylated aptamer was prepared in Tris buffer [final concentration: 20 mM (pH 7.4)], heated to 95°C for 5 min, and cooled immediately; NaCl, KCl, and $MgCl_2$ were then added to reach the final concentrations of the selection buffer. Various concentrations of cocaine (50 to 5000 nM) and a sample of 50 μM biocytin were prepared in selection buffer. Two hundred microliters of these solutions was loaded into the wells of a 96-well black flat bottom plate (Greiner). To begin the experiment, the biosensors were first immersed in buffer to obtain a baseline reading for 120 s and then immersed in 100 nM aptamer solution for 300 s, followed by quenching in biocytin solution for 60 s. The biosensors were then immersed in the buffer to establish a stable baseline for 360 s, and then challenged with cocaine solutions for 300 to 2400 s, depending on their association kinetics. Last, the sensors were immersed in the buffer to measure dissociation kinetics for various amounts of time (300 to 2400 s). Control experiments were performed by immersing aptamer-modified biosensors in a buffer during the association step rather than cocaine. Control data from reference sensors were used to remove biosensor drift and other artifacts from sample measurements. Data processing and analysis were performed using the Sartorius Analysis Kit 13. To obtain binding kinetic parameters, the data were fitted using a global fitting model available in the analysis software. "Kinetic K_d" was obtained via global fitting of sensorgrams. "Steady-state K_d" was determined by fitting the plot of sensor response at equilibrium against target concentration with the Langmuir-Hill equation. The reported error values represent the error of fit.

BLI experiments involving thrombin aptamers were performed at 37°C using the same general protocol with certain modifications. Specifically, standard streptavidin sensors were used, the thrombin selection buffer contained 7.5 μM BSA, the aptamer loading concentration was 3 nM with a loading time of 240 s, the thrombin concentration was 0.5 to 20 nM with an association time of 1000 s, and dissociation was performed for 600 s in selection buffer containing the respective non-biotinylated aptamer at a concentration of 1 μM to preclude target rebinding at the sensor surface. Control data from reference sensors (aptamers challenged with buffer) were used to remove biosensor drift and other artifacts from sample measurements. Data processing and analysis were performed using the Sartorius Analysis Kit 13. Binding kinetic parameters, kinetic K_d, and steady-state K_d were determined as described above. The reported error values represent the error of fit.

Supplementary Materials

This PDF file includes:

Figs. S1 to S43
Tables S1 to S22
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